# ENZYMATIC ANALYSIS OF G- AND V-AGENTS AND THEIR DEGRADATION PRODUCTS

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## ABSTRACT

We are presenting a rapid and highly reliable analytical methodology for nerve agents and their degradation products. The approach is to augment existing analytical methods with the specificity of the enzymatic degradation of the agents and their phosphonate ester products. The nerve agents can be hydrolyzed to their respective methylphosphonate alkyl ester (h-agent) products by alkali treatment or by specific hydrolytic enzymes, such as organophosphorus hydrolase (OPH) and organophosphorus acid anhydrolase (OPAA). A bacterial phosphonate ester hydrolase enzyme (PEH) would further degrade h-agents to methylphosphonic acid (MPA). The methodology is based on using these specific enzymes in two different schemes: 1) rapid screening for MPA (e.g. MS/MS) after the sample treatment with all three enzymes and 2) thorough analysis of the agent by creating a unique "fingerprint" from each agent through the appropriate enzymatic treatment. Initially, the agent would be identified and quantified (e.g. GC-FPD) from the "fingerprint" consisting of the original agent and silylated enzymatic degradation products, h-agent and MPA, by comparing the unique retention times of each analyte. The agents and their products can be further interrogated by the existing instrumental methods (e.g., LC/MS, GC/MS, and GC/MS/MS) for their eventual identification. Furthermore, since PEH can degrade alkyl and aryl esters of other phosphonates besides MPA esters, approach can be expanded to develop the capability to analyze potential novel threat OP CWAs and their degradation products. For this purpose, we are currently developing databases of the GC-FPD and GC/MS profiles for selected alkyl phosphonic acids and their alkyl esters.

## INTRODUCTION

The existing methodologies for identification of phosphonate ester based organophosphorus (OP) chemical warfare agents (CWAs) and their degradation products rely on multi-faceted analytical data integration. They are inferential, time consuming, non-generic, and need expensive/extensive instrumentation. They fail to rapidly detect and identify known G- and V-agents, not to mention the detection and analysis of unknown threat agents. In addition, because of the labile nature of these neurotoxins, a rapid detection and analysis method is also needed for the agents' phosphonate ester degradation products, which are important markers and biomarkers.

We are developing methods that utilize biotechnology-derived systems to augment existing technologies for improved methodology of the analysis of the agents and their phospho-products. The

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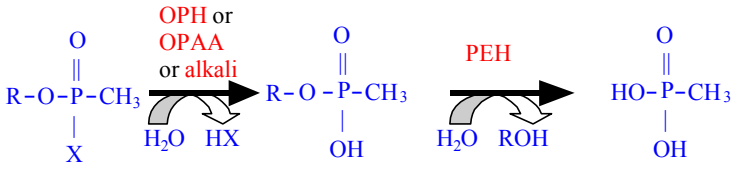
focus of the present study is to utilize naturally produced and cloned enzymes of microbial origin in order to enhance the scope, speed, and reliability for the detection and analysis of both known and prospective threat agents. Here, we are presenting a rapid and highly reliable methodology for the analysis of sarin (GB), soman (GD), cyclosarin (GF), VX, and Russian VX nerve agents, and their degradation products. In addition, we are pursuing the effort to further the methodology for the identification and analysis of prospective threat OP CWAs. The approach is to augment existing analytical methods with the specificity of the enzymatic degradation of the agents and their phosphonate ester products.

## RATIONALE

Phosphoric triester hydrolase enzymes, such as organophosphorus hydrolase (OPH) (Dumas, 1989, 1990) and organophosphorus acid anhydrolase (OPAA) (Cheng, 1993; DeFrank, 1991), hydrolyze nerve agents to their respective methylphosphonate alkyl ester (h-agent) products that are very stable. Similar transformations can also be accomplished by alkali treatment.

We recently discovered that h-agents in turn are effectively degraded by a bacterial phosphonate ester hydrolase enzyme (PEH) to methylphosphonic acid (MPA) (Elashvili, 2000, 2001). The nerve agent enzymatic degradation pathway is depicted in Scheme 1. PEH demonstrated specificity towards OP CWA products, since it effectively degraded alkali-treated products of GB, GD, GF, VX, and Russian VX nerve agents, but not the products of three similarly treated OP pesticides tested.

**Scheme 1. Enzymatic degradation pathway of G- and V-agents.**




| | | |
|---|---|---|
| GB | X = F | R = i-Propyl |
| GD | X = F | R = Pinacolyl |
| GF | X = F | R = Cyclohexyl |
| VX | X = $S(CH_2)_2N[CH(CH_3)_2]_2$ | R = Ethyl |
| R-VX | X = $S(CH_2)_2N(CH_2CH_3)_2$ | R = i-Butyl |

This unique discovery revealed new approaches to greatly improve the speed and reliability of the analytical methodology of these neurotoxins and their products. While untreated nerve agent analysis can be time consuming and ambiguous, utilizing OPH/OPAA and PEH together will produce MPA that can be rapidly analyzed (e.g. MS/MS). In another scenario, the reliability of the existing analytical methodology for these agents can be markedly improved if the analysis of the enzyme-treated samples can be included in the protocol together with the untreated agents. In this case, besides using these enzymes together to produce MPA, the utilization of only OPH/OPAA produces intermediate methylphosphonate alkyl esters from the nerve agent; thus generating two additional analytes from each agent.

The anticipated sample compositions before and after different combinations of OPH/OPAA and PEH enzymatic treatment are depicted on table 1.

Table 1. Expected sample compositions before and after OPH/OPAA and PEH treatments

| NONE | OPH/OPAA | PEH | OPH/OPAA/PEH |
|---|---|---|---|
| Agent | MPE | Agent | MPn |
| Agent + MPE | MPE | Agent + MPn | MPn |
| MPE + MPn | MPE + MPn | MPn | MPn |
| MPn | MPn | MPn | MPn |
| MPE | MPE | MPn | MPn |
| Agent + MPn | MPE + MPn | Agent + MPn | MPn |
| Agent + MPn + MPE | MPE + MPn | Agent + MPn | MPn |

The differences in the properties of the different agents and their products can be exploited to generate a unique "fingerprint" from each OP CWA. For example, the distinct retention indices on the chromatography columns and volatility characteristics of the agents and their products allow separation by gas chromatography (GC) or liquid chromatography (LC) (see Fig. 1).

We utilized GC together with a flame photometric detector (FPD) equipped with phosphorus filter (to monitor only phosphorus containing compounds) for the analysis of OP CWAs and their tri-methyl silylated (TMS) products. Each step of the enzymatic degradation can be monitored by GC-FPD as

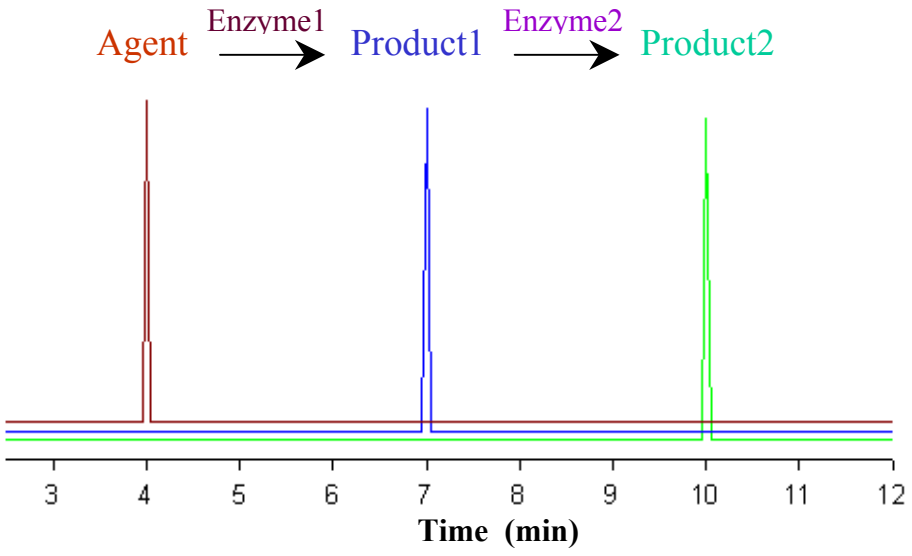



Figure 1. Representation of an agent-specific analyte "fingerprint" generated by sequential enzymatic treatment.

exemplified by the PEH transformation of hydrolyzed GF (h-GF) to MPA (Figure 2).

The unique retention times ($t_r$) can be used to tentatively identify OP CWAs and their products. For this purpose we have developed the retention times database for the OP CWAs and their silylated products for GC-FPD (Table 2).

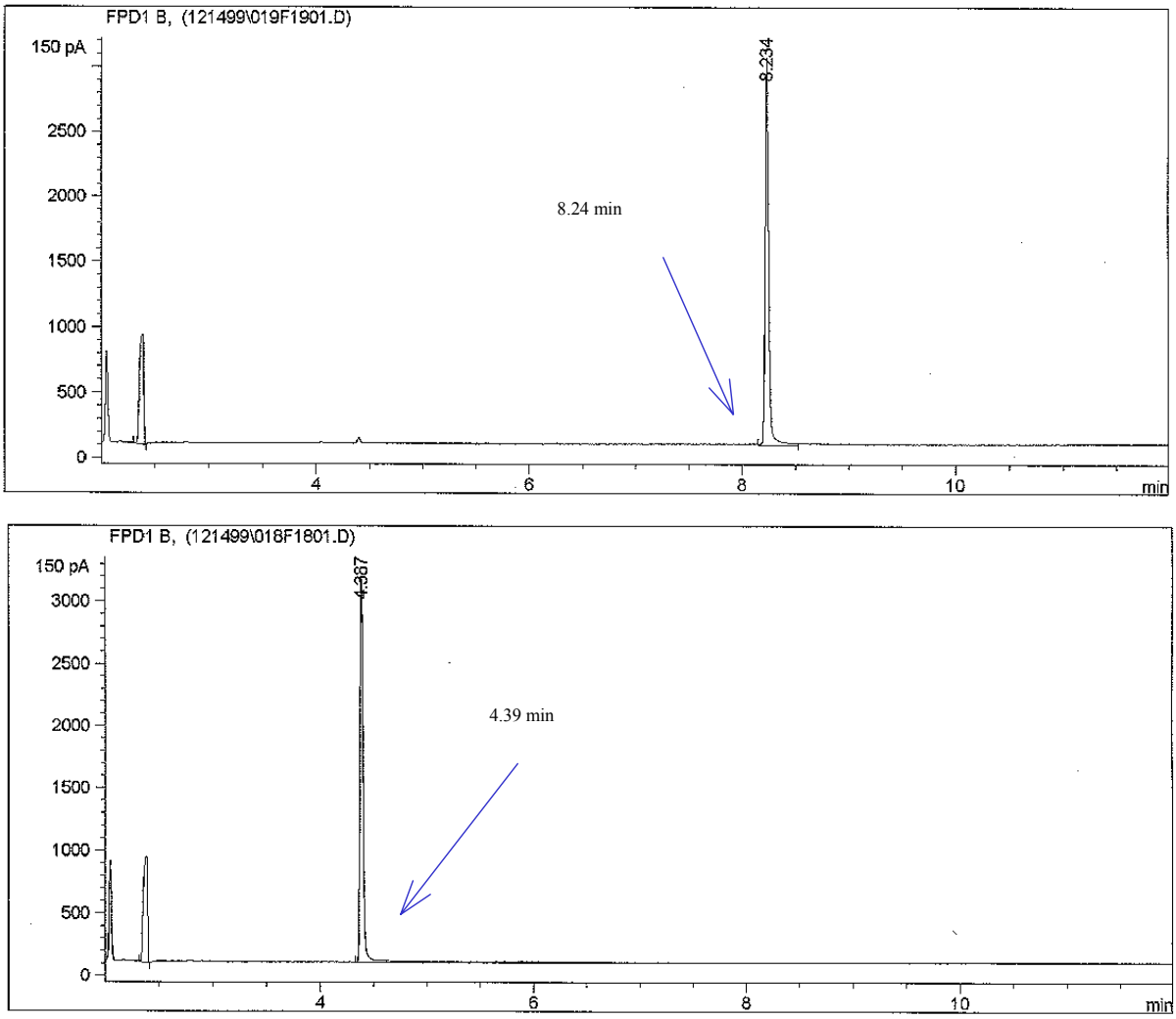



Figure 2. Retention time shift indicates PEH degradation of h-GF on GC-FPD**.** (top) h-GF without and (bottom) with PEH treatment (TMS derivatives).

Table 2. The retention times ($t_r$) of the agents and their silylated products on GC-FPD

| Agent Name | $t_r$ Values (ca. min.) | | |
|---|---|---|---|
| | Agent | h-Agent | MPA |
| GB | 4.9 | 4.0 | 4.4 |
| GD | 7.5 | 6.3 | 4.4 |
| GF | 9.2 | 8.2 | 4.4 |
| VX | 9.9 | 3.8 | 4.4 |
| R-VX | 9.7 | 4.7 | 4.4 |

Therefore, utilizing the outlined enzymatic approach would provide useful additional tool to employ for sample analysis of nerve agents. The benefit of this approach is further emphasized if samples to be analyzed contained both nerve agent and non-CWA OP. Figure 3 illustrates the basic approach for utilizing the information from tables 1 and 2 for the identification of GF agent on GC-FPD in a sample that has been heavily contaminated with non-CWA OP. In this hypothetical example, the appearance of the ~4.4 min peak corresponding to methylphosphonic acid (trimethyl silyl [TMS] derivative) and the concomitant disappearance of the ~8.2 min peak corresponding to cyclohexyl methylphosphonic acid (TMS derivative) from OPH/OPAA/PEH treated sample (panel **c**) that was present in OPH/OPAA treated sample (panel **b**) would suggest the presence of h-GF in OPH/OPAA treated sample. Similarly, the disappearance of the ~9.2 min peak corresponding to GF in the OPH/OPAA treated sample that was present in the untreated sample (panel **a**) and the concomitant appearance of the ~8.2 min peak corresponding cyclohexyl methylphosphonic acid (TMS derivative) after silylation (panel **b**) would indicate the presence of GF in the untreated sample. This example clearly illustrates the advantage for the initial identification of OP CWAs and their degradation phospho-products in samples. The agents and their products can be further interrogated by the existing instrumental methods (e.g., LC/MS, GC/MS, and GC/MS/MS) for their eventual identification.

The identification of prospective OP CWAs with existing methods poses an almost insurmountable problem because of the tri-substitution at the phosphorus atom and the enormous number of the possible novel agents that can be developed by the adversaries by replacing the existing groups on the phosphorus with various alternatives. The utilization of PEH can facilitate the identification and quantification of these potential OP CWAs and their products. We found that PEH can degrade alkyl and aryl esters of other phosphonates besides MPA esters. Therefore, using the degradation pathway similar to the one outlined for the known nerve agents can be successfully applied for the identification of the prospective OP CWAs and their degradation products as well. In this case, a $t_r$ shift of an alkali- (or OPH/OPAA) treated phospho-compound on GC-FPD, as the result of PEH treatment, would be indicative of an OP

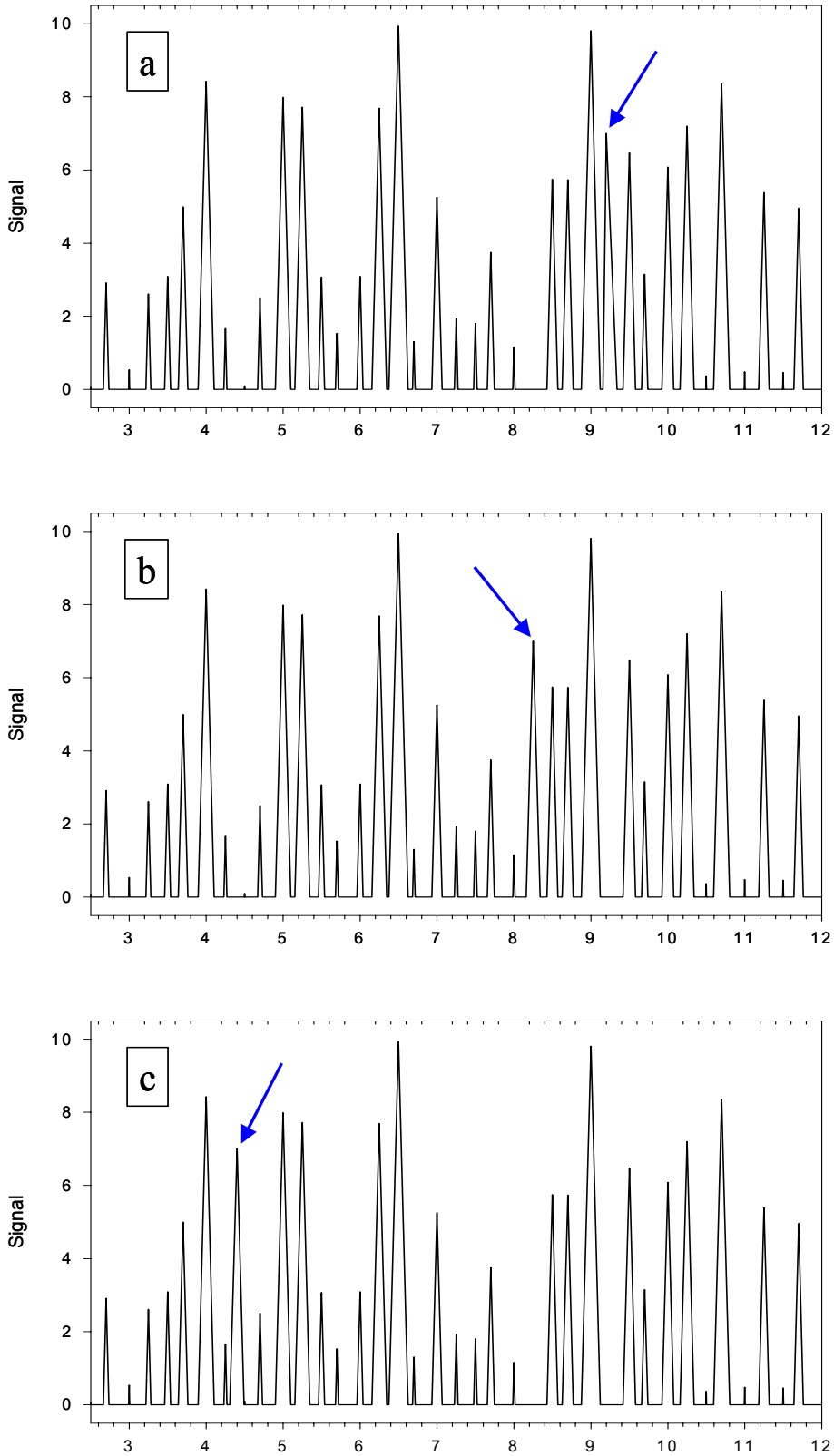



Figure 3. Representation of anticipated GC-FPD chromatograms of a hypothetical sample containing GF agent and non-CW phospho-compounds before and after enzymatic treatment. a) untreated sample, b) after OPH/OPAA, c) after OPH/OPAA/PEH.

CWA or its hydrolysis product in the original sample. To facilitate the identification of both known and novel OP CWAs and their degradation products, we are currently developing databases of the GC-FPD and GC/MS profiles for selected alkyl phosphonic acids and their alkyl esters.

## CONCLUSION

We have demonstrated a novel approach that augments existing methodology for the rapid and highly reliable identification and analysis for the known OP CWAs that is predicated by enzyme specificity. In addition, the procedure enables the analysis of the phosphonate ester products of G- and V-type agents' hydrolysis, which are important markers. Furthermore, the approach can be expanded to develop the capability to identify and quantify potential novel threat OP CWAs and their degradation products.

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